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Additional value of red blood cell parameters in predicting uncommon α-thalassemia; experience from 10 years of α-globin gene sequencing and copy number variation analysis

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Abstract

Introduction: The diagnosis of rare forms of α-thalassemia requires laborious genetic analyses. Accurate sample selection for such evaluation is therefore essential. The main objectives of this study were to investigate the predictive power of red blood cell parameters to detect rare forms of α-thalassemia (substudy 1), and to explore the frequency of rare versus common forms of α-thalassemia in our sample population (substudy 2).

Methods: In substudy 1, we reviewed all blood samples selected for extended α-hemoglobinopathy evaluation at our laboratory during 2011–2020 ($n = 1217$), which included DNA sequencing and/or copy number variation analysis. We assessed α-thalassemia positive samples at different levels of mean corpuscular hemoglobin (MCH) alone and in combination with results for red blood cell count (RBC) or red cell distribution width (RDW). In substudy 2, we examined the distribution of α-thalassemia genotypes for all samples submitted to a first-tier hemoglobinopathy evaluation at our laboratory during 2014–2020 ($n = 6495$).

Results: In substudy 1, both RBC and RDW added predictive value in detecting rare forms of α-thalassemia in samples from adults and children. In adult samples with MCH ≤ 23 pg, the presence of erythrocytosis increased the detection rate from 27% to 74% as compared to non-erythrocytosis, while normal RDW increased the detection rate from 36% to 86% as compared to elevated RDW. In substudy 2, rare forms of α-thalassemia were detected in 12% of α-thalassemia positive samples.

Conclusion: Initial assessment of MCH, RBC, and RDW provided valuable predictive information about the presence of rare forms of α-thalassemia during hemoglobinopathy evaluation.

KEYWORDS
alpha-thalassemia, erythrocyte count, erythrocyte indices, genetic techniques, thalassemia

1 | INTRODUCTION

The hemoglobinopathies comprise a diverse group of inheritable monogenic disorders resulting from defective hemoglobin synthesis. Structural defects give rise to hemoglobin variants, while quantitative defects cause thalassemia.[1] α-Thalassemia results from defective α-globin production, most commonly caused by deletions of one or more of the α-globin genes. α^0-Thalassemia refers to deletion or inactivation of both α-globin genes on a single chromosome, whereas α^+-thalassemia refers to deletion or inactivation of one of the two genes.[2] Clinically, α-thalassemia shows a wide phenotypic spectrum ranging from silent carrier states to disease incompatible with life, unless advanced transfusion therapy is initiated in utero.[3] Between these two extremes, the α-thalassemia phenotype shows varying degrees of microcytic anemia and hemolysis.

Diagnosing α-thalassemia is required to explain hematological abnormalities or to identify couples at risk of having children with severe α-thalassemia, thereby offering the parents reproductive choices. Antenatal screening programs for thalassemia have been available for several years in many countries with high disease prevalence.[4,5] However, as the hemoglobinopathies mostly originated in tropical and sub-tropical regions, these diseases are rare in the indigenous people of Northern and Western Europe.[6] Thus, health care services in these countries have had little experience with diagnosing hemoglobinopathies in general, and even less with diagnosing α-thalassemia in particular. From the 1970s onward, the population demographics changed with immigration from areas endemic in hemoglobinopathies, such as Southeast-Asia, Africa, and the Middle East.[7] Today, 9.0% of the population in Norway is of African or Asian descent.[8] Despite this fact, no national hemoglobinopathy screening program is yet established in Norway and several other low-prevalence countries. The consequence is that a hemoglobinopathy diagnosis often depends on the individual health practitioner's awareness of this particular disease group and the investigation level as determined by the laboratory.

This study was carried out at the Department of Medical Biochemistry at Oslo University Hospital, which performs approximately 1500 hemoglobinopathy evaluations annually. Our laboratory hemoglobinopathy work-up differentiates between first-tier and second-tier evaluations, generally in accordance with international recommendations.[9] First-tier evaluations include complete blood count (CBC), hemoglobin pattern analysis, and multiplex gap-polymerase chain reaction (gap-PCR) analysis[10] to detect seven common α-thalassemia deletions. However, the standard gap-PCR analysis can only detect the seven specific deletions it was designed to detect. Furthermore, carrier states with mild forms of α-thalassemia cannot be detected by hemoglobin pattern analysis. Thus, second-tier genetic analyses, including DNA sequencing and copy number variation (CNV) analysis, are required to detect less common α-thalassemia mutations and hemoglobin variants.[9] As these genetic analyses are generally labor intensive, accurate sample selection for second-tier evaluations promotes expedient resource utilization.

The hematological profile of thalassemia may resemble both iron deficiency and anemia of chronic disease, which makes the sample selection for second-tier hemoglobinopathy evaluation challenging. The variation in erythrocyte size, measured as coefficient of variation and reported as red cell distribution width (RDW), may improve the initial classification of anemias from CBC results.[11] Several researchers have previously proposed mathematical formulas based on red blood cell parameters to distinguish thalassemia from iron deficiency.[12,13] However, these studies are very heterogeneous with respect to patient selection criteria, and types of thalassemia included, and cut off-values for the parameters used. In this study, we aimed to investigate whether inclusion of red blood cell count (RBC) and RDW in addition to mean corpuscular hemoglobin (MCH) could optimize sample selection for second-tier evaluation for α-thalassemia (substudy 1). To obtain an overview of the frequencies of rare molecular defects relative to those detected by gap-PCR, we described the distribution of α-hemoglobinopathy genotypes among samples received during a 7-year period (substudy 2).

2 | MATERIALS AND METHODS

We retrospectively collected analytical results from hemoglobinopathy evaluations requested by clinicians and performed at our department. No further analyses specific for this study were carried out. The trial was approved by the hospital Data Protection Office, reference number 2014/3620, which also waived the need for informed patient consent. We reviewed results for 1217 samples in substudy 1 and for 6495 samples in substudy 2.

Our first-tier hemoglobinopathy evaluation included (1) hemoglobin pattern analysis by high-performance liquid chromatography (Variant/Variant II, β-thalassemia short program; Bio-Rad Laboratories, Hercules, CA) and/or capillary electrophoresis (Capillarys 3 Tera/Capillarys 2/Capillarys 2 Flex Piercing, Hemoglobin kit; Sebia, Lisses, France), (2) examination of seven common α-thalassemia deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, $--^{MED}$, $--^{FIL}$, $-(\alpha)^{20.5}$ and $--^{THAI}$) by gap-PCR[10] and (3) evaluation of CBC including hemoglobin concentration, RBC, MCH and RDW (measured as coefficient of variation, RDW-CV) in blood (XE-2100/XN-9000; Sysmex Corporation, Kobe, Japan or Cell-Dyn Sapphire; Abbott, IL). Furthermore, (4) iron status was evaluated based on plasma or serum ferritin concentration (Modular E170/Cobas 8000 e602/Cobas e801; Roche Diagnostics, Mannheim, Germany or Advia Centaur; Siemens Healthcare GmbH, Erlangen, Germany) and C-reactive protein (Cobas Integra 800/Modular P/Cobas 8000 c702; Roche Diagnostics, Mannheim, Germany). For some of the samples, a complete or partial first-tier evaluation was carried out at the referring laboratory by use of different measurement methods specific to the referring laboratory.

Our second-tier hemoglobinopathy evaluation included (1) α-globin gene sequencing by use of the Sanger method[14] and/or (2) CNV analysis of the α-globin gene cluster by real-time PCR.[15,16] We classified sequence variants according to ACMG Standards and Guidelines.[17] Variants classified as pathogenic or likely pathogenic were grouped as α-thalassemia positive, whereas variants classified as benign, likely benign, or of uncertain significance were grouped as α-thalassemia negative.

There were no strictly defined eligibility criteria for second-tier evaluation in our hemoglobinopathy work-up routine. On a case-by-case basis, one or more laboratory physicians reviewed clinical information and analytical results from first-tier evaluation and assessed

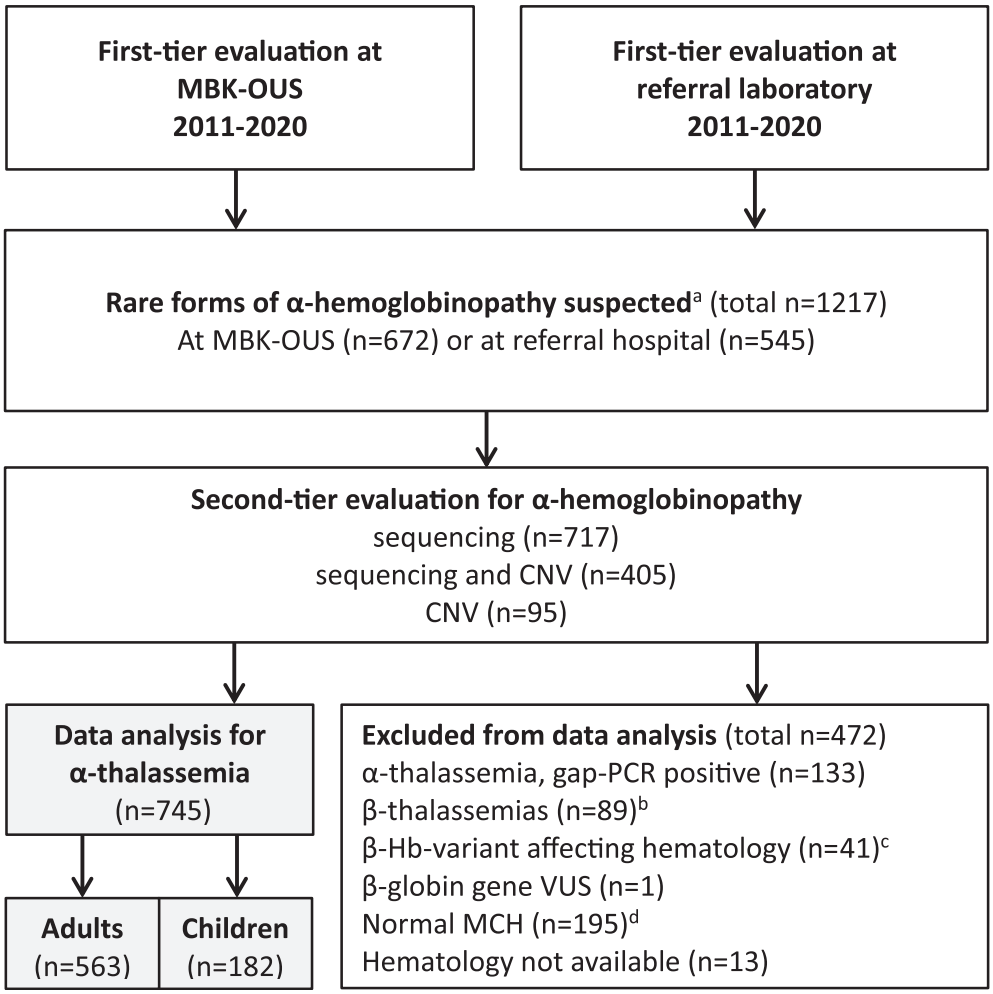

FIGURE 1 Flowchart for samples in substudy 1. [a]Rare forms of α-hemoglobinopathy were suspected in samples with unexplained hematological changes or unknown hemoglobin variants after first-tier evaluation. [b]β-Thalassemias also include patients with εγδβ-thalassemia ($n = 1$), γδβ-thalassemia ($n = 3$), Hb Lepore ($n = 2$), deletional δβ-thalassemia ($n = 2$), and combined non-deletional β-thalassemia and δ-thalassemia or δ-variant ($n = 7$). [c]Hb E (heterozygous = 20, homozygous = 8), Hb C (heterozygous = 8), Hb S (homozygous = 2), Hb SC ($n = 2$), and other ($n = 1$). [d]In adults, normal MCH means MCH ≥ 27 pg. In children, normal MCH means an MCH value less than 3 pg below patient median or higher MCH values. CNV, copy number variation analysis; MBK-OUS, Department of Medical Biochemistry at Oslo University Hospital, Norway; MCH, mean corpuscular hemoglobin; VUS, variant of uncertain significance

the indications for further investigations. A second-tier evaluation was usually carried out if any of the following criteria were present: (1) reduced MCH unexplained by either gap-PCR positive α-thalassemia, β-hemoglobinopathy, iron deficiency, or other diseases; (2) a hemoglobin pattern analysis indicating a β-thalassemia minor diagnosis, but with more pronounced hematological changes than would be expected, suggesting an increased number of α-globin genes, or (3) an unidentified hemoglobin variant assumed to result from a mutation in one of the α-globin genes. Consequently, samples with normal hemoglobin fractions in combination with either MCH values within reference ranges or obvious iron deficiency, generally would not proceed to a second-tier evaluation unless the clinical situation necessitated such investigation. Examples of such situations include pregnancies in which α-thalassemia had been diagnosed in the partner, and cases of polycythemia or hemolytic anemia for which other and more common causes had been excluded.

TABLE 1 MCH medians in different age groups derived from a dataset containing 5230 MCH values obtained from clinical routine

Age	MCH median (pg)
<1 week	35
1 week	34
2–3 weeks	33
1 month	31
2 months	29
3–4 months	28
5–8 months	26
9 months–1 year	25
2–4 years	26
5–7 years	27
8–15 years	28
16–17 years	29

Abbreviation: MCH, mean corpuscular hemoglobin.

2.1 | Substudy 1

To study the predictive value of first-tier hematological parameters in detecting α-thalassemia during second-tier investigation, we reviewed all samples submitted to second-tier α-hemoglobinopathy evaluation at our laboratory in the period 2011–2020 ($n = 1217$). These samples had been submitted for first-tier evaluation at our laboratory or at referral laboratories. We recorded the following parameters for the

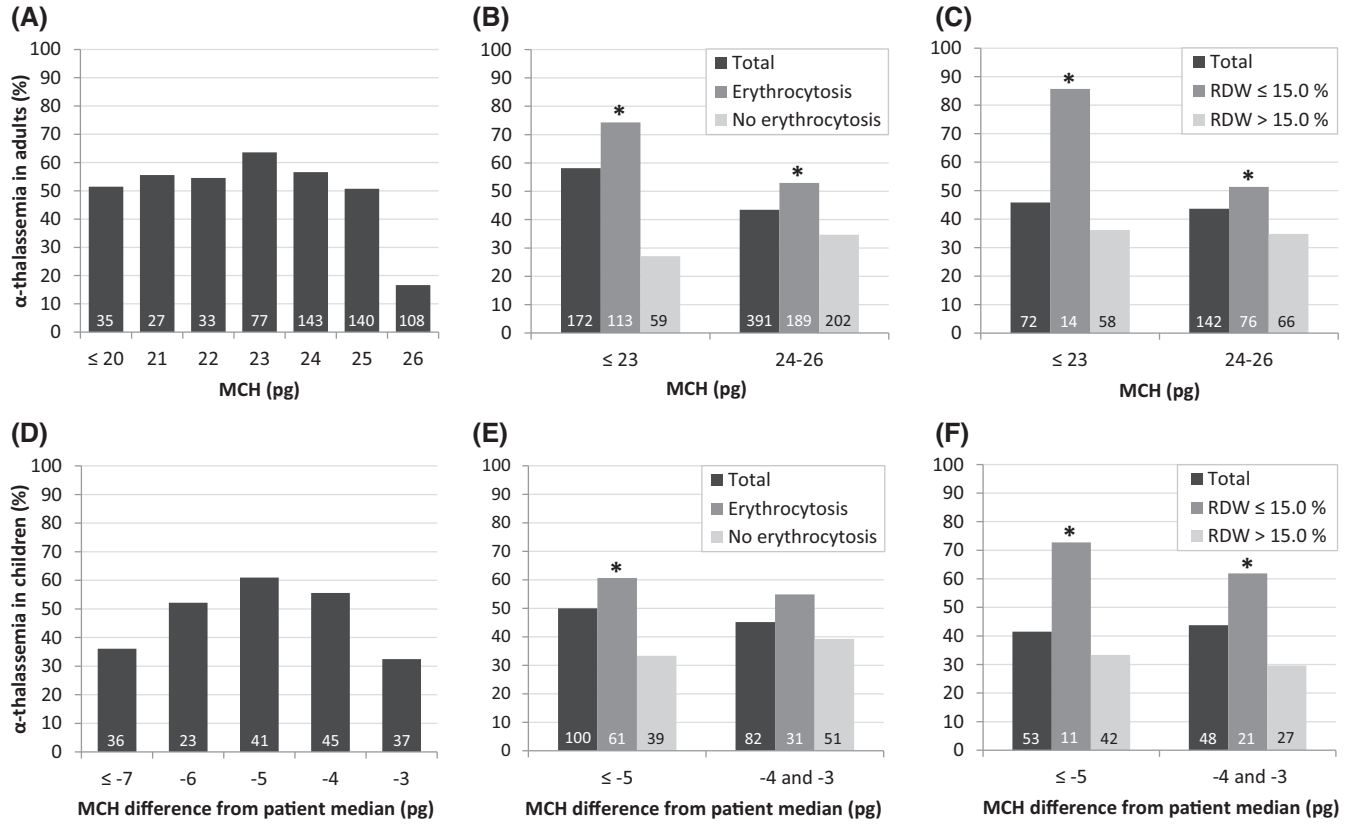

FIGURE 2 α-Thalassemia data analysis in substudy 1. The columns show the percentage of samples in which rare forms of α-thalassemia were detected (adults: A, B, and C; children: D, E, and F). Black columns represent total groups, only defined by MCH level. Dark- and light gray columns represent separate subgroups of the total group based on erythrocytosis (B and E) or RDW (C and F). The number at the bottom of each column indicates total number of samples in the particular group. Asterisks (*) denote significant differences between subgroups ($p < .05$) calculated by chi-Square test. Erythrocytosis, red blood cell count above upper reference limits by age (0–4 weeks > 6.5×10^{12}/L, 1–5 months > 5.4×10^{12}/L, 6 months–11 years > 5.3×10^{12}/L, women ≥12 years > 5.2×10^{12}/L, men ≥12 years > 5.7×10^{12}/L). MCH, mean corpuscular hemoglobin. Medians for children are detailed in Table 1. RDW, red cell distribution width, measured as coefficient of variation

samples: age, gender, MCH, RBC, RDW (not available for all samples), hemoglobin fractions, as well as results from gap-PCR, sequencing, and CNV analysis.

We performed data analysis on samples with reduced MCH not explained either completely or partially by other hemoglobinopathy findings. Samples excluded from data analysis are summarized in Figure 1. For adults, we defined reduced MCH as MCH below 27 pg, which is the lower reference limit used in our laboratory.[18] For children (below 18 years of age), we defined reduced MCH as MCH values of 3 pg or more below patient medians which were calculated for different age groups from a dataset containing 5230 MCH values obtained from clinical routine samples run on a hematology instrument (Sysmex XN-9000) at our laboratory during January–March 2017 (Table 1). Subsequently, we calculated the proportions of α-thalassemia positive samples related to various MCH levels, with and without the additional impact of RBC and RDW. For the analysis of α-thalassemia in relation to MCH alone, 1 pg-intervals of MCH were used according to common rules for rounding numbers. To obtain a sufficient number of samples in each group, we applied wider MCH intervals representing either mild or moderate to severe MCH reduction when studying the additional predictive values of RBC and RDW. The cut-off values for erythrocytosis[18] and RDW were set at the current reference limits used in our laboratory. The non-deletional sequence variants that we classified as pathogenic or likely pathogenic are shown in Supplementary Table S1.

2.2 | Substudy 2

To study the distribution of α-hemoglobinopathy genotypes, all samples received for first-tier evaluation at our laboratory in the period 2014–2020 were included ($n = 6495$). We excluded referred samples that had undergone first-tier evaluation at other laboratories, and samples from patients previously diagnosed with sickle cell anemia, which were solely submitted for hemoglobin fraction monitoring. Subsequently, we categorized the included samples according to the results of first- and second-tier evaluations.

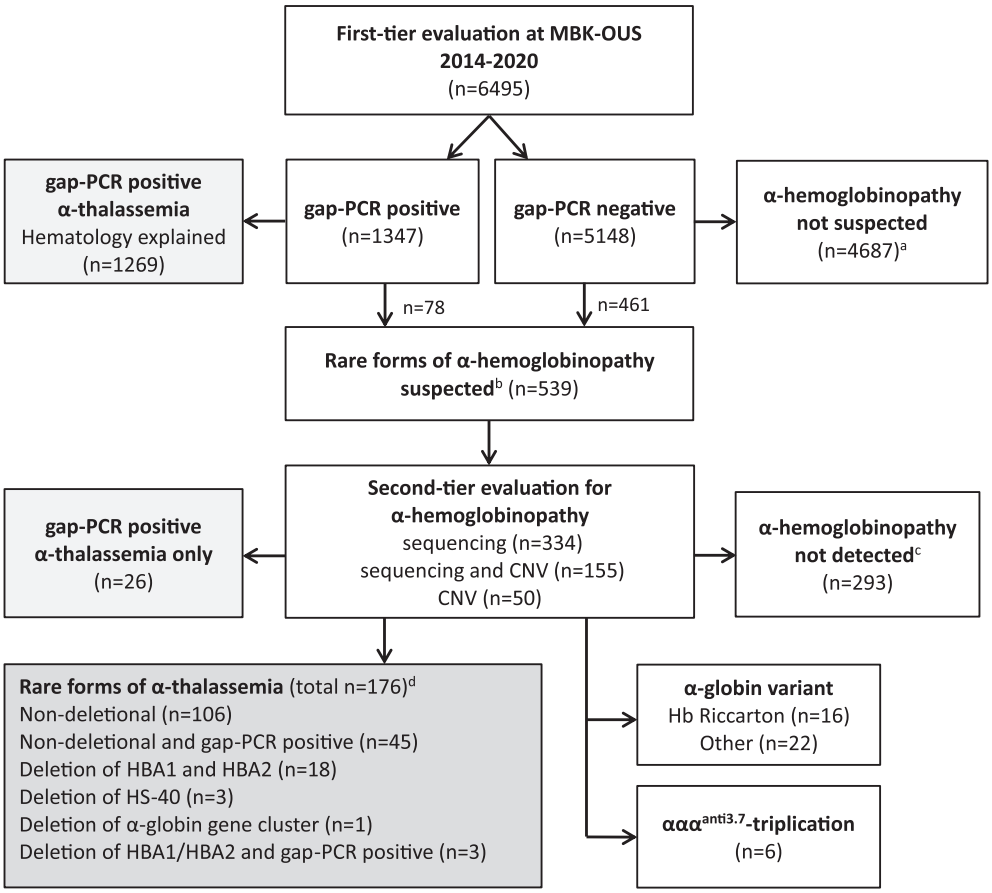

FIGURE 3 Flowchart for samples in substudy 2. Light gray boxes show α-thalassemias diagnosed by gap-PCR only. The dark gray box shows α-thalassemias diagnosed by second-tier evaluation. [a]α-Hemoglobinopathy was not suspected in samples with normal blood cell count or explained hematological changes (e.g., iron deficiency, β-thalassemia, and other diseases) and no indication of unknown hemoglobin variant. [b]Rare forms of α-hemoglobinopathy were suspected in samples with unexplained hematological changes or indication of unknown hemoglobin variants after first-tier evaluation. [c]This group consists of samples with no sequence variants detected and samples with sequence variants categorized as benign, likely benign, or variant of uncertain significance (group 1–3 according to ACMG Standards and Guidelines[17]). [d]This group consists of samples with sequence variants categorized as pathogenic or likely pathogenic (group 4 and 5 according to ACMG Standards and Guidelines[17]). Detailed results are presented in Table 2. CNV, copy number variation analysis; MBK-OUS, Department of Medical Biochemistry at Oslo University Hospital, Norway

2.3 | Statistics

We calculated MCH patient medians and Chi-square tests using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp. Armonk, NY). All other calculations were performed using Microsoft Excel 2010 (Microsoft Corp. Redmond, WA).

3 | RESULTS

3.1 | Substudy 1

To study the power of hematological parameters in predicting rare forms of α-thalassemia, we reviewed data for all samples that were submitted to second-tier hemoglobinopathy evaluation at our laboratory during the 10-year study period 2011–2020 (Figure 1). A total of 1217 samples proceeded to second-tier evaluation for α-hemoglobinopathy due to unexplained hematological changes or unidentified hemoglobin variants. Of these, 745 samples without a first-tier diagnosis associated with MCH reduction were included in the final data analysis (Figure 2). The remaining 472 samples were excluded according to details outlined in Figure 1.

Figure 2A shows the proportion of α-thalassemia positive samples at different MCH values in adults. We found α-thalassemia in 51%–64% of samples with MCH values of less than 26 pg. In comparison, the α-thalassemia proportion in samples with MCH of 26 pg was markedly lower (17%). Figure 2B,C shows the proportion of α-thalassemia positive samples in adults at different MCH values with the additional impact of RBC and RDW, respectively. Inclusion of RBC

TABLE 2 Overview of rare forms of α-thalassemia detected in substudy 2

DNA sequence variant	Additional Gap-PCR deletion	Number (*n*)
HBA2:c.95+2_95+6del		33
HBA2:c.427T>C (Hb Constant Spring)		12
HBA2:c.*94A>G		12
HBA2:c.*92A>G		9
HBA1:c.43T>C (Hb Evanston)		6
HBA2:c.326C>A (Hb Bleuland)		4
HBA2:c.-59C>T		4
HBA2:c.*93_*94del		2
HBA2:c.89T>C (Hb Agrinio)		3
HBA1:c.358C>T (Hb Groene Hart)		3
HBA2:c.2T>G		2
HBA2:c.60del		1
HBA1:c.179G>A (Hb Adana)		2
HBA2:c.345del		1
HBA1:c.300+1G>A		1
HBA2:c.428A>T (Hb Kinshasa)		1
HBA1:c.96-1G>A		1
HBA1:c.95+4A>G		1
HBA2:c.95+2_95+6del	$-\alpha^{3.7}$	9
HBA2:c.427T>C (Hb Constant Spring)	$-\alpha^{3.7}$	9
HBA2:c.*92A>G	$-\alpha^{3.7}$	2
HBA1:c.95+1G>A	$-\alpha^{3.7}$	2
HBA1:c.237del	$-\alpha^{3.7}$	2
HBA2:c.*94A>G	$-\alpha^{3.7}$	1
HBA2:c.60del	$-\alpha^{3.7}$	1
HBA2:c.1A>G	$-\alpha^{3.7}$	1
HBA2:c.2del	$-\alpha^{3.7}$	1
HBA2:c.98T>G (Hb Rotterdam)	$-\alpha^{3.7}$	1
HBA1:c.179G>A (Hb Adana)	$-\alpha^{3.7}$	1
HBA1:c.118_120del (Hb Taybe)	$-\alpha^{3.7}$	1
HBA2:c.2T>C	$-\alpha^{3.7}$	1
HBA2:c.104T>C (Hb Bass Hill)	$-\alpha^{3.7}$	1
HBA1:c.43T>C (Hb Evanston)	$-\alpha^{3.7}$	1
HBA2:c.69C>T	$-\alpha^{3.7}$	1
HBA2:c.427T>C (Hb Constant Spring) homozygous		3
HBA2:c.95+2_95+6del homozygous		2
HBA2:c.*93_*94del+HBA1:c.358C>T (Hb Groene Hart)		1
HBA2:c.143del+HBA2:c.314G>A (Hb Sallanches)		1
HBA1:c.95+1G>A+HBA2:c.1del		1
HBA2:c.427T>C (Hb Constant Spring)	--SEA	7
HBA2:c.429A>T (Hb Paksé)	--SEA	1
HBA2:c.391G>C (Hb Sun Prairie)	--FIL	1
HBA2:c.*92A>G	$-(\alpha)^{20.5}$	1
Rare deletions		
Deletion of both α-globin genes (*HBA1* and *HBA2*)		18
Deletion of regulatory site (HS-40)		3

TABLE 2 (Continued)

DNA sequence variant	Additional Gap-PCR deletion	Number (*n*)
Deletion of one of the α-globin genes (*HBA1* or *HBA2*)	$-\alpha^{3.7}$	2
Deletion of both α-globin genes (*HBA1* and *HBA2*)	$-\alpha^{3.7}$	1
Deletion of α-globin gene cluster		1

Note: Variant nomenclature according to the Human Genome Variation Society (HGVS) recommendations. Hemoglobin variants named according to the HbVar database.[26] Sequence variants categorized according to the ACMG Standards and Guidelines.[17]

and RDW both added predictive value for an α-thalassemia diagnosis, particularly in the group with MCH ≤ 23 pg.

Figure 2D shows the proportion of α-thalassemia positive samples relative to MCH levels in children. We found similar tendencies in α-thalassemia frequency by MCH as with adults. The combination of MCH with RBC (Figure 2E) or RDW (Figure 2F) clearly improved predictive values also in children, except for the addition of erythrocytosis in the group with mildly reduced MCH.

3.2 | Substudy 2

To study the distribution of α-hemoglobinopathy genotypes diagnosed during both first-tier and second-tier hemoglobinopathy evaluations, we examined data for all samples referred to our department for first-tier evaluation in the 7-year period 2014–2020 ($n = 6495$) (Figure 3). Out of these 6495 samples, we found 1347 (21%) to be gap-PCR positive and 5148 (79%) to be gap-PCR negative. The 1347 gap-PCR positive samples demonstrated either heterozygosity, homozygosity, or compound heterozygosity for one or two of the seven common α-thalassemia deletions: $-\alpha^{3.7}/\alpha\alpha$ ($n = 773$); $--^{SEA}/\alpha\alpha$ ($n = 233$); $-\alpha^{3.7}/-\alpha^{3.7}$ ($n = 209$); $-\alpha^{4.2}/\alpha\alpha$ ($n = 29$); $--^{FIL}/\alpha\alpha$ ($n = 28$); $--^{MED}/\alpha\alpha$ ($n = 28$); $-\alpha^{3.7}/--^{SEA}$ ($n = 19$); $-\alpha^{3.7}/-\alpha^{4.2}$ ($n = 10$); $-\alpha^{4.2}/-\alpha^{4.2}$ ($n = 7$); $-(\alpha)^{20.5}/\alpha\alpha$ ($n = 4$); $-\alpha^{3.7}/--^{MED}$ ($n = 3$); $-\alpha^{4.2}/--^{SEA}$ ($n = 2$); $-\alpha^{3.7}/--^{FIL}$($n = 1$); and $--^{THAI}/\alpha\alpha$ ($n = 1$). The gap-PCR results were considered to explain the hematological changes in 1269 (94%) of the gap-PCR positive samples. In the remaining 78 gap-PCR positive samples, hematological changes were not sufficiently explained and second-tier evaluation was therefore performed. In addition, 461 (9%) of the gap-PCR negative samples continued to second-tier evaluation. For the remainder of the gap-PCR negative samples, α-hemoglobinopathy was considered unlikely and no further investigation performed.

In total, 539 samples proceeded to second-tier evaluation. In 176 (33%) of these samples, we found mutations (other than the seven common deletions) consistent with α-thalassemia, as detailed in Table 2. Another 38 (7%) of the samples subjected to second-tier evaluation were diagnosed with α-globin variants, and 6 (1%) with $\alpha\alpha\alpha^{anti3.7}$-triplication. For the remaining 319 (59%) samples, including 26 gap-PCR positive samples, second-tier evaluation did not reveal any further mutations related to α-hemoglobinopathy.

Overall, we detected α-thalassemia in 1471 (23%) of the 6495 samples referred to our laboratory for first-tier evaluation during the years 2014–2020. Of these, 1295 α-thalassemia cases (88%) were detected by gap-PCR alone, while second-tier evaluation was required to establish the diagnosis for 176 (12%) of the samples.

4 | DISCUSSION

This study demonstrated the added predictive value of assessing RBC and RDW together with MCH for α-thalassemia detection in selected samples with reduced MCH. Although several previous studies have evaluated the ability of different red blood cell parameters to discriminate between thalassemia and iron deficiency in general,[12,13] this is, to the best of our knowledge, the first study that has applied red blood cell parameter analysis to predict rare forms of α-thalassemia in a second-tier hemoglobinopathy evaluation setting. Additionally, this study provided information about α-thalassemia genotype distribution in samples received for hemoglobinopathy evaluation at our department.

Substudy 1 aimed to improve sample selection criteria for detection of rare forms of α-thalassemia and focused on the transition from first-tier to second-tier evaluation. We retrospectively analyzed positive and negative findings from second-tier evaluations. Our clinical routine did not operate with strictly defined selection criteria for second-tier evaluations, which is a weakness of this study. However, some general selection criteria were in use, as outlined in the Material and Methods section. Because our clinical routine basically aimed at detecting all forms of α-thalassemia, including α^+-thalassemia carrier states, the general MCH limit for second-tier evaluation was set at the lower reference limit in adults. In a fraction of patients, however, heterozygous α^+-thalassemia may be associated with normal MCH, but this mostly applies to the common α^+-thalassemia deletions that are captured by gap-PCR regardless. Through our first-tier evaluation, normal MCH was observed in approximately 30% of adult patients diagnosed with an isolated $-\alpha^{3.7}$-deletion heterozygosity (own data, unpublished). Heterozygous non-deletional α^+-thalassemia, however, may be associated with reduced MCH to a greater extent.[19]

Reduced MCH was an absolute requirement for samples to be included in our data analysis for rare forms of α-thalassemia. Second-tier evaluation in samples with normal MCH was mainly performed to address other clinical questions such as hemoglobin variants or an increased number of α-globin genes. The limited number of samples that despite normal MCH proceeded to second-tier evaluation with the question of α-thalassemia were selected based on special indications, thus possessing a higher pre-test probability. Due to the heterogeneity of this group with normal MCH, we considered data analysis

for rare forms of α-thalassemia infeasible. For many samples, rare forms of α-thalassemia were combined with gap-PCR positive thalassemia or other hemoglobinopathies that contributed to hematological abnormalities. We also excluded these samples from data analysis in substudy 1 as their inclusion would have confounded the results.

In accordance with our reference ranges, reduced MCH was defined as MCH below 27 pg in adults. In children, our age-specific reference ranges for MCH are literature-based and extend over large age intervals. These stepwise intervals are only crude approximations to MCH development with age, which is continuous. Therefore, we considered the median in production data to provide a more robust estimate of age-related variation in erythrocyte size. To exclude samples with normal erythrocyte size in children, we restricted data analysis to samples with MCH ≥ 3 pg below patient median.

In adults, the probability of a second-tier evaluation detecting rare forms of α-thalassemia in unexplained MCH reduction was more than 50% for all groups with MCH ≤ 25 pg. Although the results showed a lower detection rate in the highest MCH group (26 pg), this MCH level should not be ignored for the possible presence of rare forms of α^+-thalassemia. The addition of RBC or RDW both generated significant improvements in detection rates, but did not show 100% positive or negative predictive value in any of the groups (complete data are shown in Supplementary Table S2). However, the distinction in α-thalassemia fraction between the groups was so pronounced, especially in the low-MCH groups, that we considered the evaluation of RBC and RDW as valuable when selecting samples for second-tier evaluation. Moreover, RBC and RDW are readily available from automated blood counters, which make them valuable in terms of resource utilization.

In children, the results generally showed the same trends as in adults. However, the additional predictive impact of erythrocytosis appeared to be slightly less than in adults. A possible explanation for this may be the difficulties of assessing continuously changing hematological variables by age in relation to specific cut-offs. The pubertal upregulation of erythropoiesis in boys that occurs individually at different ages may further confound the results. Nevertheless, RDW appeared to provide approximately equal predictivity as in adults.

Samples received from other parts of the country might have been a few days old before they reached our laboratory, which could potentially have affected the hematological parameters used in our data analysis. MCH and RBC are shown to be stable for up to 6 days both in healthy and thalassemic patients.[20] Since hematological counts were mainly performed within this time frame, results for MCH and RBC were likely not influenced by storage time. However, RDW increases considerably after a few days of storage at room temperature,[20] which may have caused falsely elevated RDW in some of our samples, thus reducing the number of samples registered with normal RDW. Regardless, a normal RDW value still predicted the presence of α-thalassemia in co-existing moderate-to-severe MCH reduction. Another potential limitation was that the red blood cell parameters were obtained from various hematology analyzers over a period of 10 years, which may have caused some heterogeneity in the measurements. On the other hand, the inclusion of different hematology analyzers as well as variable sample storage times may enhance transferability of the results into clinical routine, as this diversity is reflective of real-life laboratory situations.

Although ferritin concentration was routinely measured as part of first-tier evaluation, this parameter was not included in our data analysis. Several patients examined at our laboratory had iron deficiency or intercurrent diseases reducing the MCH value. In most cases, we considered it unreasonable to carry out second-tier evaluation in this setting. Except for the case of an antenatal situation, there should be sufficient time to reassess hematology parameters after iron supplementation or treatment of the disease. Instead, we recommended the referral of a new sample in case of persisting MCH reduction. Samples that were subjected to second-tier evaluation despite a low ferritin concentration had clinical information or small peaks in the hemoglobin pattern that increased the probability of detecting α-thalassemia. Due to this clearly biased sample selection, probably leading to falsely high detection rate in the low ferritin concentration group, data analysis related to ferritin level was not performed.

Our hemoglobinopathy evaluation routine aimed to detect all forms of α-thalassemia. However, the necessity of diagnosing α^+-thalassemia carrier states is debatable. These patients are usually clinically asymptomatic with no need for follow-up or treatment. The UK antenatal screening program recommends further investigation only in the case when MCH is less than 25 pg.[21] This UK program aims to identify couples at risk of Hb Bart's *hydrops fetalis* due to homozygous α^0-thalassemia and thus states that α^+-thalassemia is not usually a clinically important disorder. Classically, HbH disease results from compound heterozygosity of α^0-thalassemia and α^+-thalassemia, in which three out of four α-globin genes have lost their function. Although HbH disease is often considered a relatively mild disorder, characterized by mild-to-moderate chronic hemolytic anemia, non-deletional forms appear to be more severe than deletional forms.[22] Furthermore, phenotypic HbH disease has been reported in several cases due to homozygosity or compound heterozygosity of non-deletional α^+-thalassemia. In rare cases, even *hydrops fetalis* has been described as a result of non-deletional defects.[23,24] By ignoring non-deletional α^+-thalassemia carrier states, couples at risk of severe non-deletional HbH, or even Hb Bart's *hydrops fetalis* in the offspring may not be identified. As iron deficiency and thalassemia often exhibit similar hematological abnormalities, diagnosing mild forms of α-thalassemia as an explanation of reduced MCH may also be important to avoid unnecessary iron supplementation.

In substudy 2, we focused on the distribution of rare forms of α-thalassemia as compared to common α-thalassemia deletions detected by gap-PCR. We found rare forms of α-thalassemia in as many as 12% of the α-thalassemia positive samples. The real fraction might even be somewhat higher as second-tier evaluation was not routinely performed in patients with normal MCH or beta-thalassemia. To discover the small number of patients carrying rare forms of α^+-thalassemia despite normal MCH, second-tier evaluation would be required in a large portion of the patients, which we considered inexpedient. We probably also missed the diagnosis of rare forms of α-thalassemia in some β-thalassemia patients as we did not routinely

perform second-tier evaluation in this patient group in which a low MCH is explained by β-thalassemia. We limited second-tier evaluation to the few β-thalassemia patients whose partner information indicated α-thalassemia. This may be a reasonable approach to ensure detection of couples at risk of passing a serious α-thalassemia on to their children. In 293 samples, second-tier evaluation did not reveal any α-hemoglobinopathy. We assume that undetected iron deficiency and chronic diseases may explain the majority of these cases.

We also observed that several forms of non-deletional α^+-thalassemia occurred significantly more frequently than some of the α-thalassemia deletions captured by gap-PCR. This indicates that non-deletional α^+-thalassemia is not very rare in our ethnically heterogeneous population. Obviously, the distribution of different α-thalassemia genotypes may vary between different laboratories and countries depending on the ethnic groups from which samples are received. However, in our population, the results emphasized the need for second-tier evaluation for samples with still unexplained MCH reduction after first-tier evaluation. Deletions affecting only one α-globin gene were very rarely detected through second-tier evaluation, which implies that CNV analysis may be unreasonable in samples with just slightly reduced MCH.

In this study, we used conventional genetic methods such as Sanger sequencing and real-time PCR. Recently, next generation sequencing techniques have been established for thalassemia screening and have also proven to be a useful tool in resolving more complex cases. However, these techniques are still time consuming and are currently considered more costly because sequencing for thalassemia involves few genes.[25] Although rapid advances in genetic techniques enable simpler and more comprehensive diagnostics in thalassemia, we believe that the evaluation of hematological features and hemoglobin pattern analysis will still remain fundamental for accurate and resource efficient sample handling. In addition, the understanding of hematological abnormalities and results of hemoglobin pattern analysis relative to genotype is important to avoid misinterpretation of genetic results.

In conclusion, our results imply that a second-tier hemoglobinopathy evaluation, including α-globin gene DNA sequencing and CNV analysis, is necessary in cases of unexplained MCH reduction after first-tier investigation. Furthermore, assessment of MCH, RBC, and RDW provides valuable predictive information about the possible presence of a rare form of α-thalassemia. These hematological parameters are therefore considered a valuable set of tools when selecting samples for such evaluations.

AUTHOR CONTRIBUTIONS

Åshild Amelie Sudmann-Day and Petter Urdal conceived the original idea. All authors contributed to the design of the work. Bente Fjeld and Åshild Amelie Sudmann-Day performed the data acquisition and data analysis. Bente Fjeld, Åshild Amelie Sudmann-Day, Runa Marie Grimholt, Anne Cecilie Kjeldsen Larstorp, and Olav Klingenberg contributed to the interpretation of data. Bente Fjeld drafted the manuscript with support from Åshild Amelie Sudmann-Day and Olav Klingenberg. All authors critically revised the work and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PATIENT CONSENT STATEMENT

Data Protection Office at Oslo University Hospital waived the need for informed patient consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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